REMARKS

I. Disposition of the claims

Claims 1, 11, 19, 22, 24, 25, 26, and 27 are pending in this application; claims 11 and 26 are withdrawn and claims 1, 19, 22, 24, 25 and 27 are rejected. In this response, claims 1, 19, 22, 24, 25 and 27 are amended. Claim 28 is new. All amendments are made in a good faith effort to advance the prosecution on the merits of this case. Applicants respectfully request that these amendments above be entered and submit that these amendments and the following remarks will put the claims in condition for allowance.

II. Rejection of the claims under 35 U.S.C. §112

In the Office Action, the Examiner rejected claims 1, 19, 24, 25, and 27 under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement. In this and previous office actions the Examiner has noted a number of elements comprising the claimed invention that must be either demonstrated in the specification or well known to those skilled in the art at the time of filing the instant application. It is believed that the following elements represent those necessary to show enablement:

A. Ferritin-H is not present in adult red blood cells or in circulating plasma ferritin; it is lacking in the nucleus of non-fetal, globin-producing cells;

B. Supply of ferritin-H suppresses expression of the β-globin gene;

C. Suppression of β-globin results in renewed expression of γ-globin (fetal globin);

D. Significant replacement of β-globin with γ-globin results in the suppression of at least one symptom of the sickle cell disease; and

E. Means to deliver a ferritin-H protein into the nucleus of significant numbers of globin-producing cells are known and do not require undue experimentation.

Applicants submit that elements A, C, D and E were well known to those skilled in the art prior to the priority date of this application. Applicants further submit that element B was not known by others at that time and was discovered by the inventors, resulting in the presently claimed invention. The following addresses each of these elements in order.

A. Ferritin-H is lacking in the nucleus of non-fetal, globin-producing cells. While embryonic red cells contain FtH, circulating mature adult red blood cells do not. This is a developmental difference in humans and in all other vertebrates that is the basis of the present invention and is crucial to an understanding of the invention. The following references demonstrate that this was well known by those in the field at the time of application. First, *Dickey, LN, et al.* J Biol Chem 1987; **262:**7901-7907 (reference submitted with original Information Disclosure Statement) at p. 7901 bottom to p. 7902 top states: "At the end of erythroid maturation in the adult cell line, the ferritin concentration drops to between 1/16 and 1/800 that of the embryonic erythrocyte...; in the embryonic red cell, ferritin actually accumulates at the end of maturation...." Please note that the data presented in Fig. 1 and Table I of *Dickey et al.* are all from tadpole red cells from an early, non-adult developmental stage, i.e., these are ***embryonic/larval erythrocytes, <u>not</u> adult.*** These data do not pertain to adult bullfrog mature RBCs, which are almost devoid of ferritin. Note also that the discussion in paragraph 2, page 1905 of *Dickey et al.*, refers to ***tadpole*** red cells **<u>not</u>** adult red cells. Note that a tadpole is a

larval form, an early developmental stage of frog that contains only embryonic and larval/fetal-like erythroid cells. No adult erythrocytes are present at this developmental stage.

Also, Harrison and Arosio (*Harrison, PM, et al.* Biochim Biophy Acta 1996; **1275:**161-203 (reference submitted with original Information Disclosure Statement) specifically shows that serum ferritin contains only ferritin-L (FtL) subunits and has no FtH subunits whatsoever. The reference teaches on page 166 (bottom) to p. 167 (top) that "Ferritins isolated from mammalian tissues consist of a mixture of isoferritins [H and L] with a range of subunit compositions and iron contents (Fig.1).... No H-chain homopolymers have been isolated but ***human serum ferritin is devoid of H-chains***...." Fig. 1 (page 166) shows that ***serum ferritin is composed of 24 ferritin-L subunits and zero ferritin-H subunits***. The legend to Fig. 14 (p. 190) teaches the "Scheme of cellular iron homeostasis in vertebrates." This is a diagram of how iron content is regulated and only refers to adult erythroid cells in the ***precursor cell stage*** when they are making heme in preparation for making hemoglobin as they mature and, ultimately, lose all their ferritin before entering the circulation.

Numerous other early references can be forwarded to the Examiner if required. They all demonstrate that it was known to those skilled in the art that Ferritin-H is not present in adult red blood cells or in circulating plasma ferritin.

B. Supply of ferritin-H suppresses expression of the β-globin gene. In the Office Action dated 07/17/2003, the Examiner stated that the specification teaches that ferritin-H binds to a specific DNA sequence in the promoter of the human β-globin gene and represses transcription and translation of this gene in transfected cells. Applicants understand that the Examiner is not questioning enablement of this step. Nevertheless, it is noted that on pages 16 and 17 of the specification, as well as Figures 2, 3 and 4, Applicants disclose that they used

human K562 erythroleukemia cells as a source for isolating nuclear ferritin-H, and then used this nuclear ferritin-H to demonstrate DNA-sequence specific binding of the nuclear ferritin-H to the human beta-globin promoter *in vitro.*

To demonstrate that human ferritin-H binding to the beta-globin promoter causes repression of the gene driven by that promoter, co-transfection experiments were done using CV-1 primate cells (African green monkey kidney epithelial cells) as shown in Figures 1 and 6 of the specification and the corresponding text legends on pages 16 and 17. CV-1 cells are frequently used for transfection experiments by those skilled in the art. The experiments show that ferritin-H's action on the beta-globin promoter is to repress reporter gene expression driven by this promoter, and that mutating the binding site for ferritin-H abolishes the repression. This experiment therefore demonstrates that binding of ferritin-H to a specific site on the beta-globin promoter is required for repression. This beta-globin promoter is identical to the beta-globin promoter that drives expression of the mutated beta-globin in sickle cell disease.

C. Suppression of β-globin results in renewed expression of γ-globin. It was known by those skilled in the art that a compensatory increase in γ-globin (fetal) expression is expected when β-globin is decreased. This knowledge is evidenced by *Dover et al.* Blood, 69(4): 1109-13, 1987 (reference submitted with original Information Disclosure Statement) where they show that a reciprocal relationship exists between γ and β-globin gene expression in normal subjects and in those with high fetal hemoglobin production. (See the abstract provided herewith.)

D. Replacement of β-globin with γ-globin results in suppression of symptoms of sickle cell disease. The enclosed Sickle Cell Research for Treatment and Cure article on p.7 describes research done in the 1980's using 5-azacytidine to reactivate the production of fetal

hemoglobin after birth. When tested on patients with sickle cell disease (end of the first paragraph on p. 7) "[t]he results were dramatic in that the drug ameliorated most of their symptoms." Similarly, research using hydroxyurea to treat sickle cell patients in the 1990's resulted in reduction by half all of the following sickle cell symptoms: 1) painful episodes or crises; 2) hospitalizations for painful episodes; 3) episodes of acute chest syndrome; and 4) units of blood that patients needed to receive (see p. 8). There is no doubt that those skilled in the art understood that replacement of β-globin with γ-globin results in suppression of symptoms of sickle cell disease.

E. Means to deliver a ferritin-H protein into the nucleus of significant numbers of globin-producing cells is known to those skilled in the art. In fact, various delivery means are well known to those skilled in the art. The *Schwarze, SR, et al.* Trends in Cell Biology 2000; **10:**290-295 reference supplied by the Examiner states in the abstract:

> Several proteins can traverse biological membranes through protein transduction. Small sections of these proteins (10-16 residues long) are responsible for this. Linking these domains covalently to compounds, peptides, antisense peptide nucleic acids or 40-nm iron beads, or as in-frame fusions with full-length proteins, lets them enter **any cell type** in a receptor- and transporter-independent fashion. Moreover, several of these fusions, introduced into mice, were delivered to all tissues, even crossing the blood brain barrier. These domains thus might let us address new questions and even help in the treatment of human disease.

Also in *Schwarze et al.*, (p. 291, left col. lines 37-47), the authors explain that it is not necessary to understand the mechanism of transduction in order to use the process and that "[t]here currently appears to be no restriction on the type of molecule that can be transduced into cells when covalently attached to a PTD [protein transduction domain]: compounds, peptides,

oligonucleotides, peptide nucleic acids and full-length proteins, including antibodies, enzymes and sequestering proteins, have all been transduced[14,15,17-22]."

Schwarze et al. further teach (p. 291, middle column, line 28 to p. 292, line 5): "This protocol [using TAT-PTD fusion proteins] has been successfully used to transduce and obtain phenotypes for a variety of proteins, including p16, adenovirus E1A, human-pailloma-virus E7, caspase-3, Bid, HIV protease, IkB, Rho, RAC, CDC42, Cdk2 dominant negative, E2F-1 dominate-negative and pRB (Refs 2,1,22,27-30 ....)." Also:

> PTD-linked peptides and proteins can be transduced into cells simply by adding them to the tissue culture medium. The constructs are internalized in a rapid, concentration-dependent manner that achieves maximum intracellular concentration in less than 15 min[16,22]. Normally, final media concentrations of 25-200 nM provide biological activity, although concentrations in excess of 1 uM can be used.
>
> TAT-mediated transduction provides several advantages over DNA transfection, ***the current standard method of intracellular protein expression.*** Importantly, **all eukaryotic cell types tested to date are susceptible to transduction,** excluding yeast....Even osteoclasts and **peripheral-blood mononuclear cells (PBMCs)** [which include erythroid precursor cells]...can be effectively transduced[29,31]. Additionally, as transduction occurs so rapidly (15 min...), issues of timing can be addressed. The exact intracellular concentration can be controlled precisely just by varying the amount added to the culture medium. Furthermore, **every cell in the population** appears to contain a near identical intracellular protein level.
>
> "...TAT-mediated transduction has recently been successful for a variety of protein sizes (15-120 kDa) and biological functions...."

(*Schwarze et al.* p. 292, right-hand column, lines 7 to p. 293, left-column, line 5.) Please note that ferritin-H monomers are 21kDa and dimers are 42kDa, fitting into the proteins that have been successfully transduced.

Although Applicants disagree in part with the data interpretation in the *Broxmeyer et al.* reference cited by the Examiner in the Office Action, the reference does demonstrate that ferritin-H can be administered both *in vitro* and *in vivo* and reach the bone marrow cells. Also, on p. 5 of the Nov. 3, 2004 office action, the Examiner notes that *Meyron-Holts et al.* teach that exogenous ferritin molecule as a whole can be taken up by receptors on the surface of erythroid precursor cells. While Applicants disagree with the assumption of degradation in *Meyron-Holts et al.,* the reference clearly shows that apoferritin is taken up by the cells long enough to chelate iron, something that it cannot do from inside a lysosome, and therefore must reside for some time in the cytoplasm. Thus it appears clear that means to deliver a ferritin-H protein into the nucleus of significant numbers of globin-producing cells were known to those skilled in the art prior to the filing of this application and that specific details will not require significant or undue experimentation.

In summary, it is clear from the above discussion and literature that elements A, C, D and E above were well known to those skilled in the art prior to the priority date of this application, and that element B, the major discovery by the inventors, is adequately enabled in the specification, thus enabling the instant claims, as amended. Because claims 1, 19, 24, 25, 27 and new claim 28 comply with the enablement requirement of 35 U.S.C. 112, first paragraph, the above-referenced rejections should be withdrawn.

III. Rejection of the claims under 35 U.S.C. § 102

In the Office Action, the Examiner rejected claim 22 under 35 U.S.C. 102(b) as being anticipated by *Broxmeyer et al.* (PNAS 1991; 88:770-4). Also claims 1, 19, 22, and 27 were rejected under 35 U.S.C. 102(b) as being anticipated by *Adams et al.* (New Eng J Med 1998; 39:5: 5-11), and as evidenced by *Files et al.* (J Pediatric Hematol on col 2002; 24:284-90) and *Sowemimo-Coker* (Transfus Med Rev 2002 Jan; 16:46-60).

The Examiner reasons that *Broxmeyer et al.* teach a composition comprising a recombinant heavy-chain ferritin and administering the composition *in vitro* and *in vivo* into bone marrow cells or mice. However, the ferritin used in *Broxmeyer et al.* is merely a chemical composition designed to test a biological function mechanism, and is not a **pharmaceutical composition** comprising **a therapeutically effective amount of ferritin-H capable of binding to the promoter of a human β globin gene at -148 to -153 bp from the transcription start site** as called for in instant claim 22. Therefore, *Broxmeyer et al.* does not anticipate claim 22 as amended.

The Examiner also reasons that transfusion of blood from an adult human into a sickle cell patient as described by *Adams et al.*, and as evidenced by *Files et al.* and *Sowemimo-Coker,* anticipates claims 1, 19, 22, and 27 of the instant invention. However, the only elements of transfused adult blood that contain even minute amounts of ferritin-H are the very, very small amounts of ferritin-H within the small amounts of 'housekeeping' ferritin within some of the white blood cells - amounts that are far below the amount of ferritin-H needed to repress HbS production and to activate HbF production and far below the therapeutically effective dosage called for in the claims as amended. As explained above, no significant dosages of ferritin-H are being delivered with transfused red cells or with serum ferritin, and therefore the references relied upon by the Examiner do not anticipate the instant claims.

As a result, amended claims 1, 19, 22, and 27 are not anticipated by the references relied upon by the Examiner. Accordingly, the rejections under 35 U.S.C. 102(b) of claims 1, 19, 22, and 27 should be withdrawn.

CONCLUSION

Applicants respectfully submit that the pending claims 1, 19, 22, 24, 25, 27, as amended, and new claim 28 are allowable. Applicants respectfully request that these claims be passed to issuance. Should the Examiner have any questions, comments, or suggestions in furtherance of this application, the Examiner is invited to contact the attorney of record by telephone, facsimile, or e-mail at the Examiner's convenience.

This is intended to be a complete response to the Office Action mailed on July 12, 2006.

I hereby certify that this correspondence is being deposited in the United States Postal Service as first class mail in an envelope addressed to: Mail Stop Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450, on the date shown below.

Barbara Krebs Yuill

Date: Dec 12, 2006

Respectfully submitted,

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U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
National Institutes of Health
National Heart, Lung, and Blood Institute

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U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
Sickle Cell Research for
Treatment and Cure
Research
Treatment
Cure
NATIONAL INSTITUTES OF HEALTH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

Sickle Cell Research for Treatment and Cure

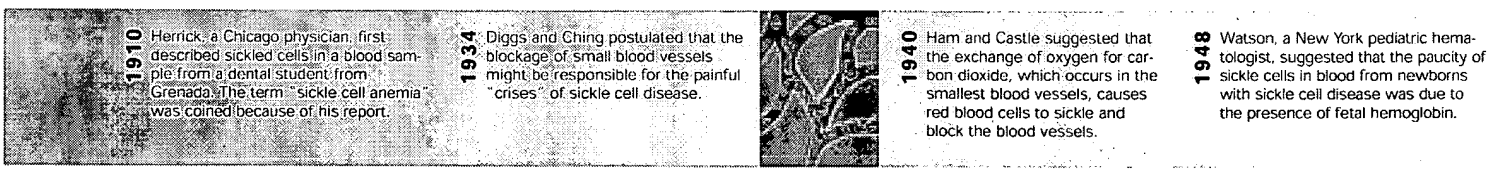
Introduction

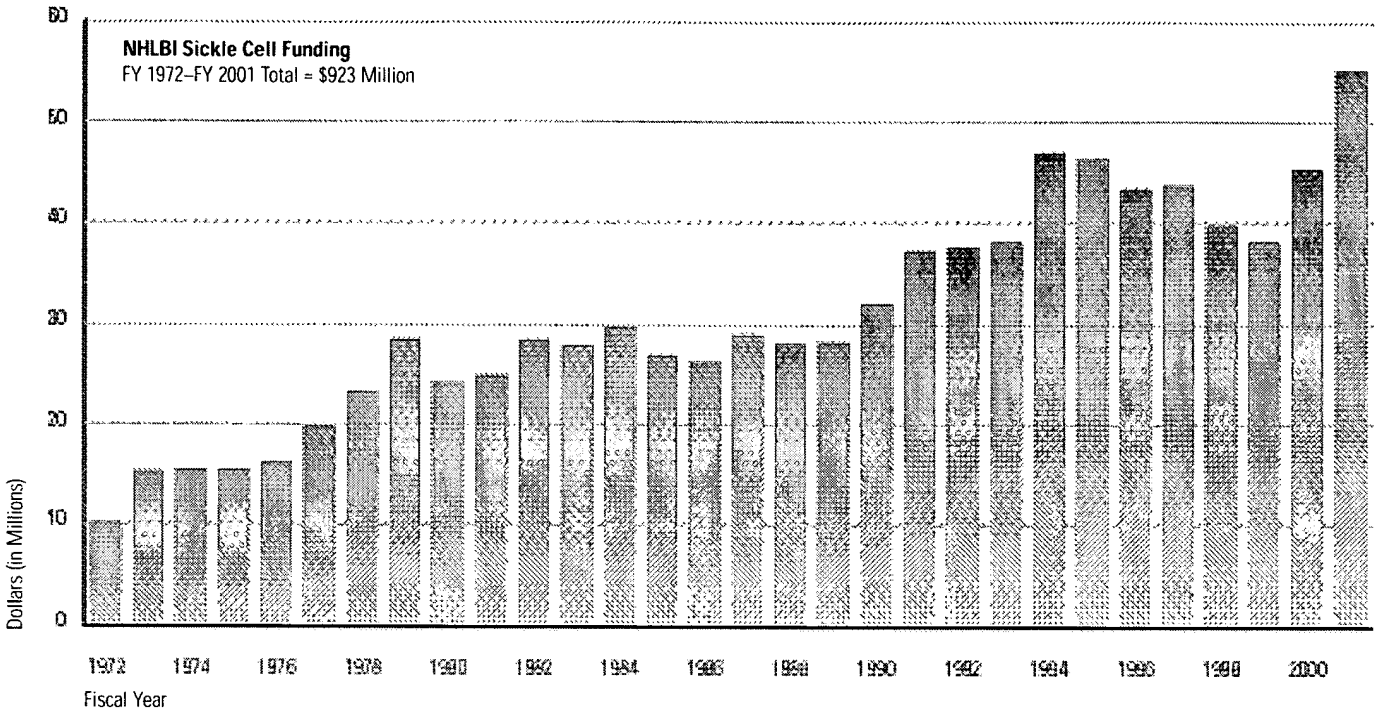
In his "Special Message to the Congress Proposing a National Health Strategy" of February 18, 1971, President Nixon made research on sickle cell anemia a national priority:

> "A . . . targeted disease for concentrated research should be sickle-cell anemia - a most serious childhood disease which almost always occurs in the black population. It is estimated that one out of every 500 black babies actually develops sickle-cell disease.
>
> "It is a sad and shameful fact that the causes of this disease have been largely neglected throughout our history. We cannot rewrite this record of neglect, but we can reverse it. To this end, this administration is increasing its budget for research and treatment of sickle-cell anemia . . ."

Legislation was subsequently introduced in both the House and the Senate to provide for the control of sickle cell anemia, and only 14 months later, on May 16, 1972, the National Sickle Cell Anemia Control Act (P.L. 92-294) was signed into law. P.L. 92-294 provided for the establishment of voluntary sickle cell anemia screening and counseling programs; information and education programs for health professionals and the public; and research and research training in the diagnosis, treatment, and control of sickle cell anemia.

Shortly after the act was passed, the Secretary of the Department of Health, Education, and Welfare responded by establishing a National Sickle Cell Disease Program and by assigning to the National Heart, Lung, and Blood Institute (NHLBI, then the National Heart and Lung Institute) the responsibility for developing and supporting a program of research in sickle cell disease and for coordinating the overall program. The extent of the Institute's commitment to improving the lives of persons with sickle





Since 1972, the NHLBI has invested over $923 million in sickle cell disease research.

cell disease is reflected in the size of its research investment, which totaled $923 million during the years 1972–2001.

The return on this public investment in sickle cell disease research has been impressive. Unlike the situation 30 years ago, babies born today with sickle cell disease have available to them a number of treatment options that can mediate, and in some cases even prevent, complications of the disease. They also have before them the promise that research will continue to increase our understanding of the disease and its complications, to expand and improve upon the options for treatment, and to develop a safe and effective cure.

Sickle Cell Disease

Sickle cell disease is a genetic blood disorder that affects about 72,000 Americans, predominantly those of African ancestry. It occurs when a child inherits from each parent

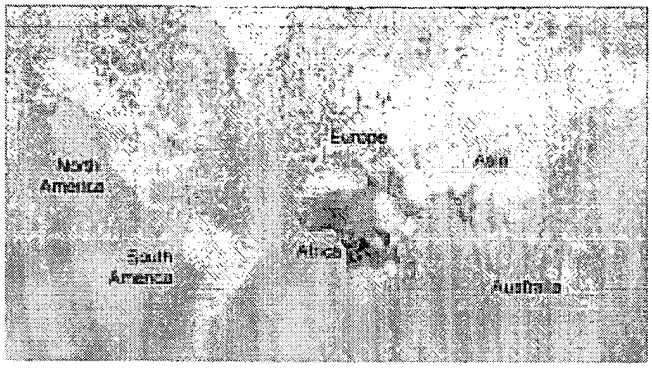
1949 Pauling, Itano, Singer, and Wells demonstrated that the abnormality of sickle cell disease resides in the hemoglobin protein molecule, thereby establishing the concept of a "molecular disease."

1949 Neel and Beet independently described the inheritance of sickle cell anemia.

1952 Tosteson noted abnormalities in how ions are transported into and out of sickle cells.

1953 Conley and Schneider independently developed a diagnostic tool to identify various forms of sickle cell disease and other conditions due to defective hemoglobin.

1954 Allison demonstrated the protective effect of sickle cell trait against malaria, explaining the high gene frequency of sickle hemoglobin in individuals from areas in which malaria was endemic.



Many Africans and people of African descent have the gene for sickle hemoglobin.

Red blood cells containing sickle hemoglobin can assume a variety of distorted shapes.

Harvesting the Fruits of Earlier Research

Here are some examples of how basic laboratory results from years ago have provided a basis for lifesaving advances against sickle cell disease and offer hope for future progress.

- Early laboratory tests that could distinguish blood from people with sickle cell disease and other blood disorders served as a foundation for the widespread screening of newborns. (See page 4, "The CSSCD and PROPS—Identifying a Problem, Finding a Solution.")
- Awareness of the existence of multiple types of hemoglobin (e.g., epsilon and zeta [embryonic] hemoglobins; gamma [fetal] hemoglobin; alpha, beta, and delta [adult] hemoglobins), and realization that both gamma and beta hemoglobin are capable of binding with alpha hemoglobin to form the complete, four-part hemoglobin protein that allows red blood cells to carry oxygen, paved the way for treatments based on reactivation of fetal hemoglobin synthesis. (See page 6, "Hope Through Fetal Hemoglobin.")
- A basic understanding of blood typing and transfusion medicine enabled patients with sickle cell disease to benefit from prophylactic and therapeutic transfusions. (See page 9, "Stopping Stroke.")
- Experiments in the 1950s demonstrating that mice whose bone marrow had been destroyed by irradiation could be rescued via infusions of bone marrow from healthy mice constituted one step in a chain of discoveries that set the stage for bone marrow transplants to cure human diseases. (See page 10, "Transplantation.")

a gene that makes an altered form of hemoglobin, the protein that carries oxygen throughout the body. This genetic alteration often causes the red blood cells to assume the crescent or sickle shape that gives the disease its name. Unlike normal red cells that pass smoothly through the blood vessels, sickle cells tend to become entangled and to obstruct blood flow.

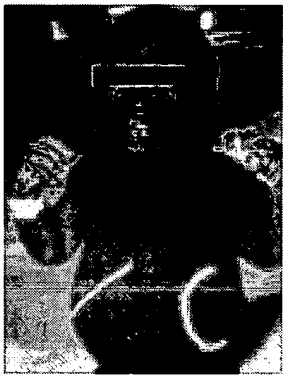
1955 Smithies developed another test to identify abnormal hemoglobin.

1957 Ingram demonstrated that the abnormality of sickle hemoglobin is a mutation at position 6 of the beta-hemoglobin protein chain.

1963 Perutz deciphered the three-dimensional structure of the hemoglobin protein. He discovered that functional hemoglobin is composed of two pairs of hemoglobin chains.

1967 Robinson and coworkers developed a technique that can distinguish among sickle hemoglobin, normal adult beta-hemoglobin, and fetal hemoglobin. Their technique is still employed in many laboratories as a confirmatory test.

1968 Bertles and Milner described red cells from individuals with sickle cell disease that remain sickled even when oxygen levels are restored. These cells are termed irreversibly sickled cells.



Sickle cell disease has affected this infant's spleen and liver.

The result is episodes of extreme pain ("crises"), as well as chronic damage to vital organs. Two of the organs most susceptible to damage are the brain and the lung, and strokes and a life-threatening respiratory problem, known as acute chest syndrome, are frequent complications for sickle cell disease patients. The disease also destroys the spleen at a very early age, which impairs the body's immune system and renders youngsters extremely vulnerable to overwhelming bacterial infections. Although there is much variability in the severity of the disease, many with sickle cell disease face a shortened life expectancy and a host of troubling, debilitating, and expensive health problems.

The CSSCD and PROPS—Identifying a Problem, Finding a Solution

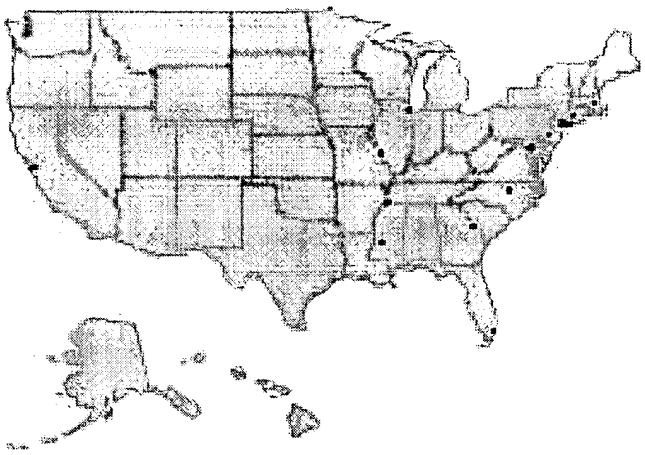
In the 1970s, federally funded research in laboratories throughout the country brought about a revolution in our understanding of sickle cell disease - it was the first human disease to be fully characterized at the DNA and RNA levels. And yet, little was known about the clinical course of the disease. The medical literature carried reports of only the sickest patients, and information about the typical course of the disease and likely response to therapeutic and preventive approaches was lacking.

The NHLBI responded to this need in 1978 by launching the multicenter, 4,000-patient, Cooperative Study of Sickle Cell Disease (CSSCD). In addition to collecting important epidemiological data, the study sites provided the basic infrastructure for one of the first major clinical trials to address sickle cell disease. Pneumococcal infection had been identified as a threat to infants and young children with sickle cell disease as early as 1966. In 1983, 12 of the CSSCD sites joined forces with 11 other centers to test the effectiveness of penicillin in preventing the deadly *Streptococcus pneumoniae* sepsis in infants and toddlers. In 1986, the study, called the Penicillin Prophylaxis in Sickle Cell Disease Study (PROPS), was terminated 8 months early because it had already produced some remarkable results; researchers found that daily administration of oral penicillin to children aged 3 months to 3 years could reduce the incidence of infection by 84 percent. The impact of the PROPS results was pronounced. They not only established a new standard of care for infants with sickle cell anemia, but also brought about a widespread change in policy regarding the screening of newborns. Although the technology for screening newborns for sickle cell disease had been available since the early 1970s, it was not generally employed because an early diagnosis offered no advantage. Without an effective treatment, nothing could be done to decrease

1970 A milder variation of sickle cell disease was found in Saudi Arabia by Ali and colleagues and was associated with increased levels of fetal hemoglobin.

1971 Nalbandian reported that intravenously administered urea ameliorated the clinical expression of sickle cell disease. His studies led to the examination of the antisickling effect of cyanate (a byproduct of urea) by Manning and Cerami. Urea was later found to be ineffective by the National Institutes of Health (NIH) Cooperative Urea Trials Group, and cyanate was found to be toxic by Peterson in 1974.

1972–73 The National Heart, Lung, and Blood Institute (NHLBI) began funding the first 15 of its Comprehensive Sickle Cell Centers.



The 23 sites of the Cooperative Study of Sickle Cell Disease were located in major metropolitan centers.

morbidity or mortality. PROPS changed all that, providing indisputable evidence that early diagnosis of sickle cell disease and the subsequent administration of prophylactic penicillin could save children's lives. Due largely to those results, the Consensus Development Conference on Newborn Screening for Sickle Cell Disease and Other Hemoglobinopathies, convened by the National Institutes of Health (NIH) in 1987, recommended that "every child should be screened for hemoglobinopathies to prevent the potentially fatal complications of sickle cell disease during infancy."

With the prestige of the Consensus Development Conference recommendations added to the strength of the

Conclusions From the NIH Consensus Statement "Newborn Screening for Sickle Cell Disease and Other Hemoglobinopathies."

(NIH Consensus Statement Online 1987 Apr 6–8 [cited 2002 Aug 15];6(9):1–22.)

1. Effective intervention in children with sickle cell disease provides a major impetus for neonatal screening. Prophylactic penicillin therapy provided in a setting of comprehensive care has been found to significantly reduce the morbidity and mortality of patients with pneumococcal sepsis.
2. Reliable, simple, and cost-effective techniques for mass screening of neonates are available and have demonstrated validity.
3. The benefits of screening are so compelling that universal screening should be provided. State law should mandate the availability of these services while permitting parental refusal.
4. Centralization of laboratory services improves efficiency and decreases the probability of error.
5. To be effective, neonatal screening must be part of a comprehensive program for the care of sickle cell patients and their families. These services must include a network of providers who ensure optimal medical care, psychosocial support, and genetic counseling. Such followup capabilities should be in place before screening is instituted.
6. Further research should focus on the following: improving and evaluating the technology for screening; defining the impact of screening on the physical, social, cognitive, and emotional development on the child and on family members; assessing other methods of management of infection; and providing optimal education of individuals and families at risk.

In summary, the panel concludes that every child should be screened for hemoglobinopathies to prevent the potentially fatal complications of sickle cell disease during infancy.

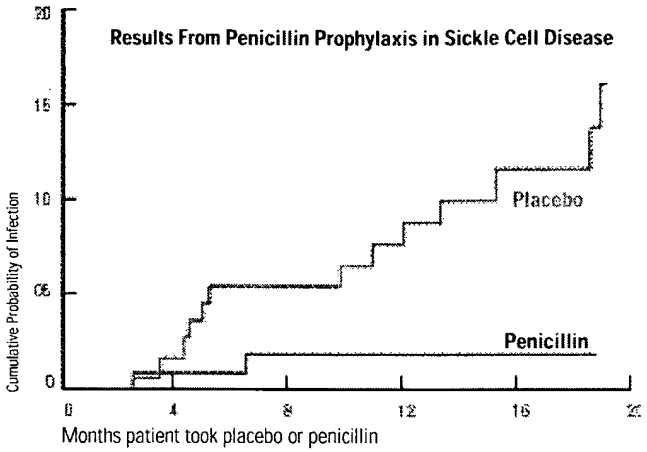
1973 Garrick and coworkers developed methods for neonatal screening employing spots of blood on filter paper.

1974 Pearson and colleagues demonstrated the feasibility of routine screening of all newborns for sickle cell disease.

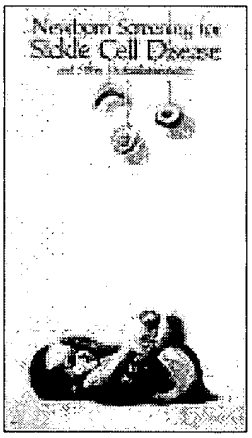
1974 Kan and associates developed a method for prenatal diagnosis by sampling fetal blood from the umbilical vein.

1976 The NHLBI invited researchers to submit grant applications on the topic "Synthesis of Fetal Hemoglobin in Human Erythroid Cells." Leading molecular biologists responded and began investigating ways that fetal hemoglobin production could be increased as a therapy for sickle cell disease.

1978 Kan and Dozy reported an approach to prenatal diagnosis of sickle cell disease using DNA obtained by chorionic villus biopsy or amniocentesis.



Results from PROPS show that 125 mg of penicillin twice daily prevents infection in children less than 3 years old.



In 1987, the NIH held a consensus development conference on Newborn Screening for Sickle Cell Disease and Other Hemoglobinopathies.

PROPS findings, the public health community quickly responded. Whereas before the Conference, fewer than 14 States offered/provided neonatal screening for sickle cell disease, today 44 States, the District of Columbia, Puerto Rico, and the Virgin Islands provide universal screening for sickle cell disease, and screening is available by request in the other 6 States.

Of course, the logical next question was, When, if ever, can we safely discontinue the penicillin regimen for those babies found to have sickle cell disease? That question was answered in 1995 by the NHLBI-sponsored PROPS II, which demonstrated that once a child reaches 5 years of age, penicillin prophylaxis can be safely stopped.

Hope Through Fetal Hemoglobin

The hemoglobin molecule in the red blood cells of children and adults differs from its counterpart in the red blood cells of fetuses. For most people, the process of replacing red blood cells that contain the fetal form of hemoglobin with red blood cells that contain the postnatal form, a process that begins around birth, is of no consequence. However, for those with sickle cell disease this replacement makes a profound difference because it is their altered postnatal form of hemoglobin that causes their red blood cells to sickle.

As early as 1948, researchers noted that the adverse effects of sickle cell disease were reduced in very young infants who continued to show circulating red cells with fetal hemoglobin. Yet it was not until decades later, when researchers supported by the CSSCD and others noted milder symptoms in those adults with sickle cell disease who had circulating blood cells containing fetal hemoglobin, that the search for ways to stimulate fetal hemoglobin production as a treatment for sickle cell disease began in earnest.

1978 The NHLBI began an epidemiological study of the natural history of sickle cell disease. The Cooperative Study of Sickle Cell Disease (CSSCD) included more than 4,000 individuals ranging in age from newborns to adults in their sixth decade. It was the first multi-institutional study designed to document prospectively the clinical course of sickle cell disease from birth to adulthood.

1979 Hoover and coworkers demonstrated that red blood cells from patients with sickle cell disease stick more readily to the endothelial cells lining the blood vessels than red blood cells from other people.

1980 The nucleic acid sequence of the human beta-hemoglobin gene was reported by Maniatis' laboratory. Smithies and Weissman reported the complete sequence of the human (fetal) gamma-globin gene.

A combination of basic research advances provided the first hint that drugs could be developed to stimulate the production of fetal hemoglobin in sickle cell disease patients. Researchers had previously determined that the genes involved in hemoglobin are located on chromosomes 11 and 16 and had characterized the pattern by which they are activated to produce hemoglobin during fetal and neonatal development. Others discovered that an anticancer drug, 5-azacytidine, was capable of reactivating the production of fetal hemoglobin after birth. Once researchers established that it stimulated the production of fetal-like hemoglobin in chickens and baboons, the NHLBI began a collaboration between investigators in its Comprehensive Sickle Cell Centers and others located at the NIH Clinical Center to determine the effect of the drug in terminally ill patients with sickle cell disease. The results were dramatic in that the drug ameliorated most of their symptoms.

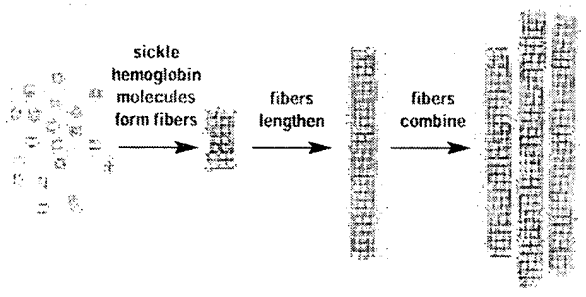
Yet, despite its favorable properties, 5-azacytidine will never be an acceptable therapy because it is also a toxic carcinogen. Fortunately, it was not the only potential stimulant of fetal hemoglobin being studied. Preliminary studies on butyrate, a fatty acid that binds to DNA and occurs naturally in humans, showed that it also had promise, although its use continues to be experimental. Meanwhile, other researchers focused on another cancer drug, hydroxyurea, that they believed could affect fetal hemoglobin production in a way similar to the effect of 5-azacytidine.

How Does Fetal Hemoglobin Help?

The basic pathogenesis of sickle cell disease is the formation of sickled cells. Within the cells, the sickle hemoglobin molecules combine to form fibers, which then combine with other fibers to form a rigid gel that is responsible for the deformation, or sickling, of the cells. Fetal hemoglobin disrupts fiber formation to the extent that a rigid gel does not form.

One study showed that a fetal hemoglobin concentration of 25 percent essentially eliminates gelling under physiologic conditions. This finding is of great clinical importance, because drugs such as hydroxyurea and butyrate may be capable of increasing fetal hemoglobin to these levels. The NHLBI also supports studies of how hydroxyurea and other agents may influence hemoglobin gene expression and how gene expression is regulated during development. A thorough understanding of how hemoglobin genes are switched on and off should lead to new therapeutic approaches that enhance fetal hemoglobin synthesis.

Understanding fetal hemoglobin's role in the gelling process also has opened other potential avenues of treatment. Researchers are working to develop compounds that interrupt the gelling process as another treatment approach for patients with sickle cell disease.

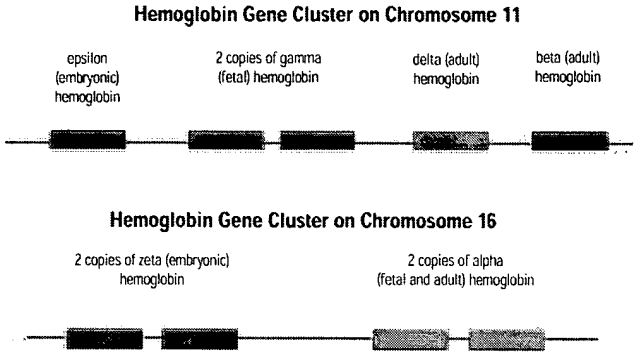


Unlike hemoglobin in children and adults without sickle cell disease, the hemoglobin of sickle cell patients sticks together and forms a fibrous gel that gives the sickled red blood cells their rigid structure.

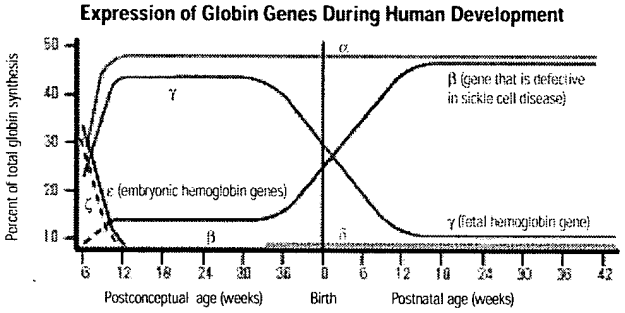
1980 Hebbel and colleagues provided evidence that binding of sickle cells to the inside of blood vessels might be sufficient to block blood flow. They further showed that the extent of stickiness might be a determinant of disease severity.

1982 DeSimone, Heller, and colleagues demonstrated that an antileukemic drug, 5-azacytidine, elevated the fetal hemoglobin levels of anemic baboons. Although clinical studies showed that 5-azacytidine produced a large increase in fetal hemoglobin in patients with sickle cell disease, the potential carcinogenicity and toxicity of the drug limited its use.

1984 Independent observations from the laboratories of Nagel, Wainscoat, Labie, Kazazian, and Orkin revealed three major DNA haplotypes associated with the beta-hemoglobin gene. These haplotypes were termed Benin, Senegal, and Bantu (Central African Republic) after the areas in which they predominate.



Multiple types of hemoglobin are found on chromosomes 11 and 16 in approximately the order in which they are expressed during development.



Hemoglobin genes are expressed at specific times during human development.

By 1991, with numerous studies indicating that hydroxyurea could indeed be a valuable treatment, the NHLBI began its Multicenter Study of Hydroxyurea in Sickle Cell Anemia (MSH). The trial, which was scheduled to run until May 1995, was brought to an end 4 months early because of the compelling results. Hydroxyurea treatment reduced by half all of the following sickle cell symptoms or complications:

- Painful sickle cell episodes or crises
- Hospitalizations for painful episodes
- Episodes of acute chest syndrome (a life-threatening condition similar to pneumonia)
- Units of blood that patients needed to receive

The MSH results figured prominently in the 1998 decision by the Food and Drug Administration (FDA) to make hydroxyurea the first agent approved for the prevention of painful episodes in adult patients with sickle cell disease.

Because fetal hemoglobin levels in most people disappear entirely after the first few months of life, researchers suspected that hydroxyurea could also help pediatric patients with sickle cell disease. But they also had a serious concern: Could hydroxyurea be safely given to children? Another NHLBI-supported study tested the safety and efficacy of hydroxyurea in patients aged 5 to 15. That study not only confirmed the efficacy of hydroxyurea in

1984 The first bone marrow transplant in a sickle cell anemia patient was reported.

1984–85 Veith and colleagues; Platt and Nathan; and Charache, Boyer, and Dover independently demonstrated that hydroxyurea and other compounds increase fetal hemoglobin levels.

1985 Smithies demonstrated that DNA sequences could be corrected inside cells.

1986 The NHLBI Penicillin Prophylaxis in Sickle Cell Disease Study reported that infants and young children placed on prophylactic penicillin had significantly lower rates of *Steptococcus pneumoniae* infections than children who received a placebo. The study established the first preventive therapy for children with sickle cell disease and resulted in a significant reduction of the major cause of death in young children.

children, showing results similar to those in adults (i.e., hydroxyurea increased fetal hemoglobin and total hemoglobin levels and decreased complications associated with sickle cell disease), but also demonstrated its safety. Hydroxyurea showed no adverse effect on growth or development.

The NHLBI is now evaluating whether hydroxyurea can benefit even younger patients. A pilot study of hydroxyurea given to children between ages 6 and 24 months demonstrated that the drug is well tolerated and stimulates fetal hemoglobin expression; an additional study is scheduled to begin this year.

Further analysis of data from MSH has shown that hydroxyurea therapy reduces health care costs. Currently, investigators are monitoring volunteers from MSH to determine how long-term hydroxyurea therapy affects their quality of life, morbidity, and mortality.

Stopping Stroke

One of the important findings of the CSSCD was that pediatric patients with sickle cell anemia have rates of stroke in the range of 0.5–1 percent per year. In an attempt to find a strategy that would prevent first-time stroke, the NHLBI initiated the Stroke Prevention Trial in Sickle Cell Anemia (STOP) to identify children between the ages of 2 and 16 who were at risk for first-time stroke and to determine whether periodic blood transfusions were more effective at preventing stroke than standard supportive care.

In September 1997, STOP followed the pattern established by PROPS and MSH, in that it too was terminated early because of favorable results. Study participants who received regular prophylactic transfusions experienced a 90-percent reduction in their stroke rate compared with those who did not. With the completion of STOP, we now know that we can effectively prevent the two leading causes of death in children with sickle cell disease: pneumococcal sepsis and stroke.

As with the prophylactic penicillin regimen, however, STOP left unanswered the logical followup questions: Must the transfusions be continued indefinitely? Could they be stopped after some period of time, and if so, when? The NHLBI is currently supporting another study, called STOP II, to address these issues.

Stroke prevention is a major reason for transfusing sickle cell patients, but it is not the only one. Transfusions are an effective treatment for patients with chronic debilitating pain, pulmonary hypertension, and anemia associated with chronic renal failure. However, chronic transfusions invariably cause iron concentrations to increase throughout the body, and high iron concentrations result in damage to vital organs, especially the liver, heart, and pancreas. The NHLBI continues to support research

1987 An expert panel convened by the NIH to discuss newborn screening for sickle cell disease recommended screening at birth for sickle cell disease for all infants born in the United States and placing all affected infants on prophylactic penicillin by the age of 3 months. Forty-four States, the District of Columbia, Puerto Rico, and the U.S. Virgin Islands presently screen for newborns with sickle cell disease.

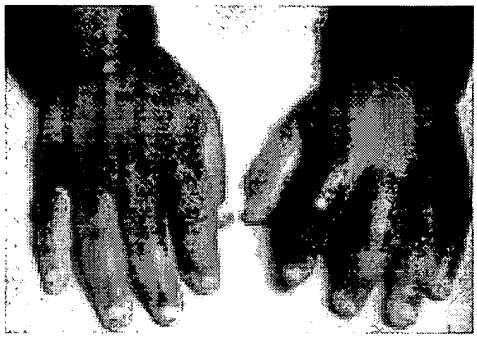
1991 Platt and the CSSCD conclusively demonstrated the major effect of fetal hemoglobin on the severity of sickle cell disease.

1992 Dover and colleagues demonstrated that fetal hemoglobin production in sickle cell disease is partially determined by a locus on the X chromosome.

Stroke Risk Factors Identified by the CSSCD, STOP, and Other Studies

All pediatric patients

- Prior transient ischemic attack
- Low steady-state hemoglobin
- Rate and recency of acute chest syndrome
- Elevated systolic blood pressure
- Stroke in a sibling
- Subtle neurological abnormalities
- Severe anemia
- High leukocyte count
- Genotype
- Transcranial doppler (TCD) ultrasound reading ≥ 200 cm/sec



Dactylitis (painful swelling of hands and feet) indicates that a child is at increased risk of pain crises, stroke, acute chest syndrome, and death.

efforts to reduce the adverse effects associated with chronic transfusion therapy by finding safe and effective ways to remove excess iron.

Transplantation

Although bone marrow transplantation had been used for some time to treat leukemias and other fatal disorders of the blood, no one had pursued it as a potential therapy for sickle cell disease until 1984, when a bone marrow transplant performed to treat a pediatric patient with leukemia was reported to have also cured the child's sickle cell disease.

That bone marrow transplantation was overlooked as a possible treatment approach for sickle cell disease is not at all surprising. It is a serious procedure that entails substantial risk to the patient. Prior to the transplant itself, patients must undergo a chemotherapeutic regimen to destroy their own bone marrow and immune system. Some patients do not survive the chemotherapy. Others suffer from life-threatening infections before their bone marrow and immune systems are sufficiently regenerated. Some bone marrow transplants simply do not work. Still others seem to work initially but then fail because the immune cells produced by the transplanted marrow attack the tissues and organs of the patient (graft-versus-host disease) or because the patient succumbs to other complications, such as hemorrhage.

1992 A large group of investigators completed the phase I trial of hydroxyurea as a therapeutic agent for sickle cell disease. Results were promising, so the Multicenter Study of Hydroxyurea in Sickle Cell Anemia (MSH) began.

1993 Brugnara and colleagues suggested clortrimazole as a therapy for sickle cell disease because of its inhibition of potassium and calcium ion channels and its subsequent reduction of sickle cell dehydration.

1995 MSH demonstrated the first effective therapy for severely affected adults with sickle cell disease: painful episodes were reduced by 50 percent.

A Summary of the Bone Marrow Transplant Process

- The patient and his or her family and physicians determine that bone marrow transplant is worth the risks.
- A suitable donor is found.
- The patient undergoes a toxic preparative conditioning regimen of chemotherapy and/or radiation to destroy existing bone marrow.
- Special precautions are taken to shield the patient from infection, since his or her immune system has been destroyed by the preparative regimen.
- The patient receives the transplant.
- In the weeks to months following the transplant but before total engraftment occurs, the patient and medical team are vigilant about preventing, detecting, and treating complications, including
  - Infection
  - Acute graft-versus-host disease
  - Bleeding
  - Damage to heart, kidneys, liver, lungs
  - Graft failure
- After patient is discharged from hospital, followup care begins. It includes treatment of
  - Long-term effects of preparative regimens (cataracts, infertility)
  - Severe fatigue, which may require treatment with medication or red blood cell transfusions
  - Chronic graft-versus-host disease
  - Side effects of medications used to treat transplant complications

Today, transplants are considered only for sickle cell patients whose disease is severe enough to justify the risk. However, the NHLBI currently is supporting a range of research programs to reduce the risk by improving the likelihood of a successful transplantation. Umbilical-cord blood is one promising area of investigation. Increasing evidence suggests that donors of umbilical-cord blood do not need to match recipients as closely as bone marrow donors do. In addition to indicating that use of cord blood may reduce the risks of rejection and graft-versus-host disease, this also suggests that transplantation of unrelated cord blood may provide a source of stem cells for children with hard-to-match tissue types. Investigators are also exploring an approach in which a patient's bone marrow is only partially destroyed before performing a transplant. This "mixed-chimerism" protocol, which

Examples of Diseases That Can Be Treated by Bone Marrow Transplant

- Acute Myelogenous Leukemia
- Acute Lymphoblastic Leukemia
- Chronic Myelogenous Leukemia
- Congenital Thrombocytopenia
- Hodgkin's Lymphoma
- Multiple Myeloma
- Myelodysplastic Disorder Syndromes
- Non-Hodgkin's Lymphoma
- Severe Aplastic Anemia
- Sickle Cell Anemia

1996 Kan and colleagues developed a method for the prenatal diagnosis of sickle cell disease from maternal blood samples.

1996 Cole-Strauss and colleagues used molecular biology techniques to correct the sickle cell mutation of the beta-hemoglobin gene in a test tube.

1996 The first multicenter study of bone marrow transplantation in children with sickle cell disease reported that the procedure can cure young sickle cell patients who have a matched sibling marrow donor.

1997 Townes and Rubin developed the first transgenic mice that had human sickle hemoglobin and human fetal hemoglobin but lacked mouse hemoglobin.

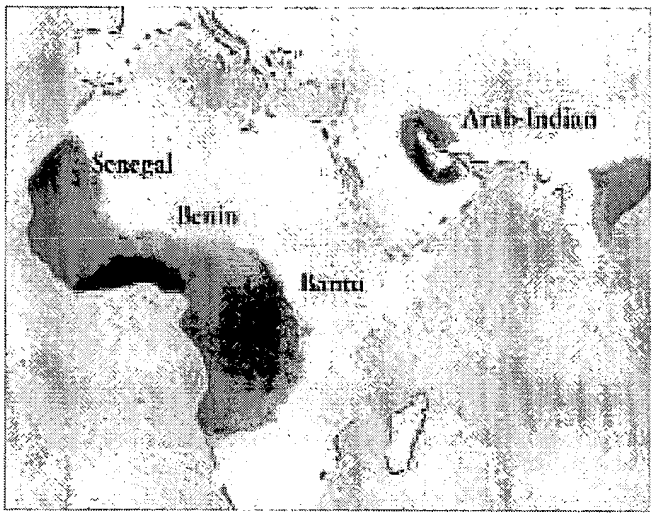
leaves the patient with components of two immune systems, would be especially attractive for children, because it would entail a less toxic chemotherapeutic regimen to be used before the transplant. It would not only further decrease the chance of death, but also reduce the likelihood of permanent infertility. Mixed-chimerism protocols are also being explored as a possible treatment approach for adults.

Clinical Severity, Genetic Modifiers, and Gene Therapy

Despite a detailed body of knowledge about sickle cell disease at the molecular level, researchers do not yet know why sickle cell patients show such extreme variability in disease severity. Individuals with sickle cell disease all have the same defect in the gene that produces beta-hemoglobin, yet some of them are subject to strokes, others are subject to frequent painful episodes, and still others show minimal symptoms.

Researchers examining the region of the chromosome *nearby* the gene for beta-hemoglobin noted variations in the sequence among individuals. The variations followed four major patterns, or haplotypes. This suggests that the genetic mutation that causes sickle hemoglobin is associated with at least four different genetic backgrounds. Recent studies have shown that clinical differences may exist among the haplotypes and that the differences may serve as predictors of disease severity. For example, people who have the Arab-Indian variation tend to have a milder form of sickle cell anemia, sometimes with no symptoms.

Some clues to the causes of variability in disease severity are provided by the beneficial effects of elevated levels of fetal hemoglobin. Elevated levels of fetal hemoglobin are associated with fewer painful crises, a reduced frequency of acute chest syndrome, a delayed onset of damage to the spleen, and a slower rate of degeneration of the hip and shoulder joints, which is another frequent complication.



The four major sickle hemoglobin haplotypes are associated with different geographic regions.

1997 The Stroke Prevention Trial in Sickle Cell Anemia (STOP) demonstrated that periodic transfusions could prevent first-time stroke in susceptible children.

1999 De Franceschi, Brugnara, and an international team of colleagues described the first clinical phase I study of oral magnesium pidolate, which prevents sickle red cell dehydration by blocking the loss of potassium.

1999 Stamatoyannopoulos, Kan, Nathan, and Nienhuis developed a strategic plan for improved treatment and cure.

In 2001, the NHLBI began an effort to stimulate research examining the genetic elements that modulate the severity of sickle cell disease. Identification of other, as yet unknown, genetic modifiers of the clinical severity of sickle cell disease may help physicians determine which pediatric patients are likely to experience more severe disease and initiate targeted therapies to extend and improve the quality of their lives.

For years, one of the greatest impediments to research on sickle cell disease was the lack of a useful animal model of the disease. This hurdle was overcome in 1997, when two groups of NHLBI-supported investigators inserted the human gene responsible for sickle cell disease into mice, thereby creating transgenic models of the human disease. The two models differ slightly; one produces both human sickle hemoglobin and human fetal hemoglobin and displays mild symptoms, whereas the other, which produces only sickle hemoglobin, closely mimics severe sickle cell disease.

That advance is critical to researchers who are seeking a gene-therapy cure for sickle cell disease. Their ultimate objective is to correct the defective gene and then insert it into the bone marrow of sickle cell patients to stimulate the production of normal hemoglobin. Recent experiments show promise. In December 2001, NHLBI-supported scientists announced that they had corrected sickle cell disease in two mouse models of the disease using gene therapy. Scientists are hopeful that their approach can be applied to human patients by removing some of a patient's own bone marrow cells, genetically correcting them, and then infusing the corrected cells back into the patient.

Deformed Membranes and Dehydrated Cells

A large body of evidence suggests that abnormalities of the red cell membrane contribute to the pathophysiology of sickle cell disease. The defective hemoglobin in sickle cell disease also weakens the membranes of the red cells and ultimately causes them to fail from repeated sickling. The changes in cell shape that occur during sickling include the appearance of long protrusions from the red cell surface. Unlike their normal counterparts, the hemoglobin molecules in sickle cell disease tend to bind to one another, especially after they have delivered their oxygen to the surrounding tissues. Investigators have observed that as they bind together, they push through the membrane, causing it to deform and then fail. These alterations of the membrane during sickling increase the tendency of the red cells to adhere to the linings of blood vessels. Clinical investigators have begun to evaluate membrane-active drugs as possible treatments for patients who have sickle cell disease. Agents that have been studied include clortrimazole, magnesium compounds, glutamine, and omega-3 fatty acids. Since not all patients

2000 Moore, Charache, and the MSH investigators published cost-effectiveness data showing that hydroxyurea therapy is cheaper than standard care for adult patients with sickle cell anemia. The decrease in aggregate costs for hospitalized crises, blood transfusions, and acute chest syndrome more than compensated for the increased cost of frequent monitoring of blood counts for patients taking hydroxyurea.

2000 A multicenter group at 30 sites reported that acute chest syndrome (a respiratory complication associated with sickle cell disease) is commonly precipitated by fat embolism and infection, especially community-acquired pneumonia. Adult patients with neurologic symptoms frequently developed respiratory failure. Aggressive treatment with transfusions and bronchodilators improved oxygenation and allowed most patients with respiratory failure to recover.

A Framework for the Future: Towards Improved Treatment and Cure

The NHLBI has adopted and already begun to implement a strategic plan for progressing to improved treatments and ultimately a cure. It consists of two components:

Improved treatment

- Facilitate the development of more effective inducers of fetal hemoglobin
- Test the new inducers of fetal hemoglobin in large animals
- Initiate carefully devised clinical investigations with human patients of those approaches found to be safe and effective in animal studies

Gene therapy for a cure

- Facilitate the development of improved technologies for hemoglobin gene transfer
- Test the new gene transfer approaches in large animal studies
- Initiate carefully devised clinical investigations with human patients of those approaches found to be safe and effective in animal studies

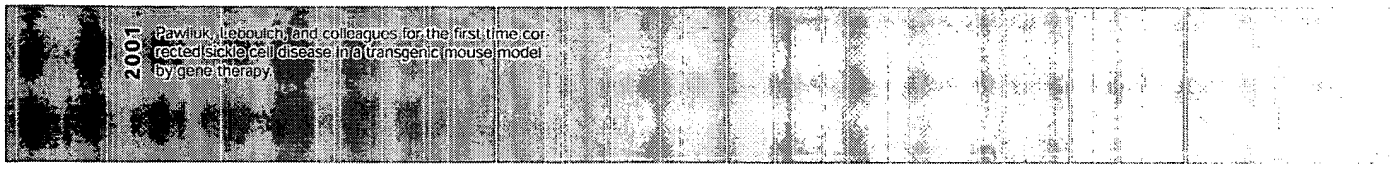
respond to or can take hydroxyurea, it is hoped that these alternatives will lead to new approaches to treat patients with sickle cell disease.

Red blood cells have a finite lifespan; as they fail, they must be replaced. Sickle cell patients have unusually high concentrations of newly formed red cells, known as reticulocytes, because their red blood cells have a shorter lifespan than normal red blood cells. In sickle cell disease, reticulocytes not only are inordinate in number, but also have unique properties that make them particularly susceptible to dehydration. As red cells become dehydrated, their concentration of hemoglobin increases, thereby increasing the proximity of hemoglobin molecules to one another so that they have more opportunity to bind together. In addition, the dehydrated cells are much less flexible. They are, therefore, more likely to lodge in small blood vessels, where they contribute to such characteristic manifestations of sickle cell disease as pain crises. An increased understanding of the dehydration process has led to pilot clinical studies of potential therapeutic agents to regulate red blood cell hydration.

A Disease of the Blood Vessels Too?

Like their red blood cells, the blood vessels of sickle cell patients differ from those of people who produce normal hemoglobin. One of the most prominent differences is that endothelial cells from patients with sickle cell disease appear to enter into the circulation at an unusually high rate. In addition, mere contact between the red blood cells and the endothelial cells is sufficient to stimulate an immune response in sickle cell patients. The immune response causes an inflammation, which further reduces the space available for blood to flow and thereby increases the likelihood of blockage formation.

Findings related to blood vessel constriction in general also have applicability in sickle cell disease. Much research has been conducted over the past several years on nitric oxide and its role in regulating blood pressure.

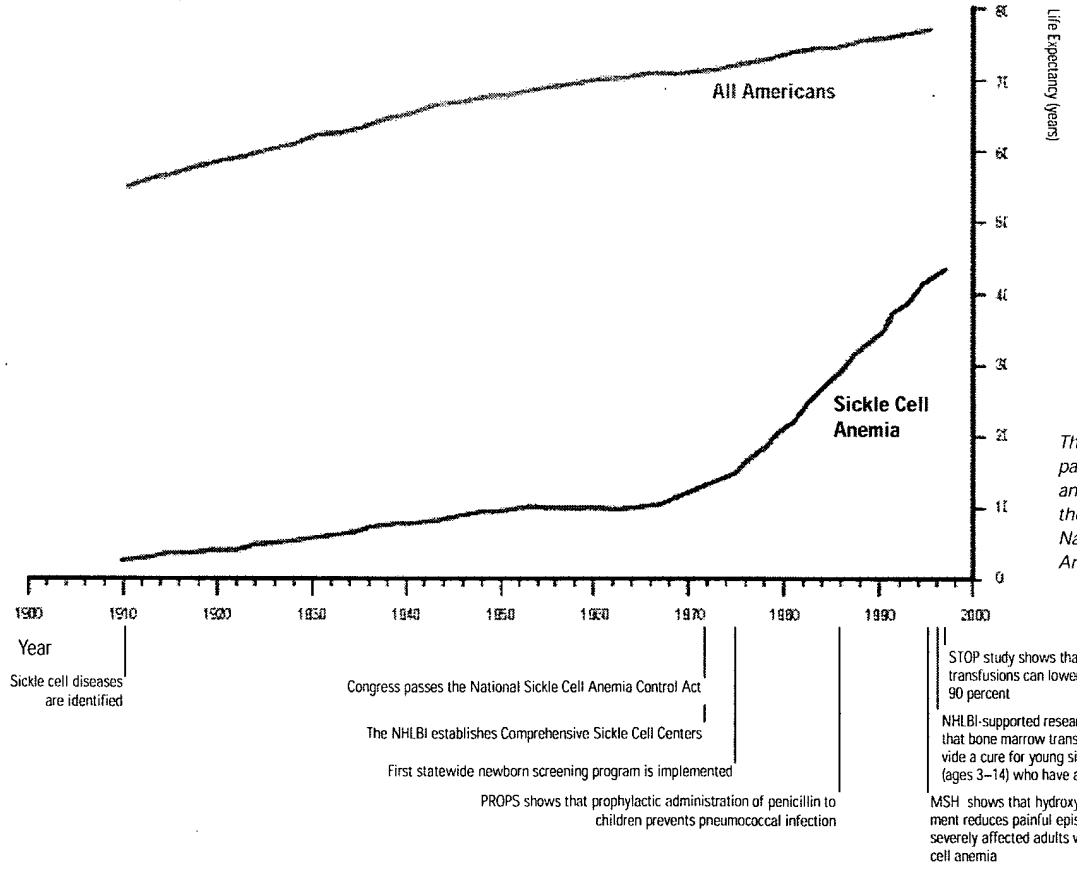


One of its functions is to keep blood vessels open so that blood can flow freely; nitric oxide also suppresses the proteins that help blood cells to stick to the vessel walls. These observations, combined with the knowledge that patients with sickle cell disease have abnormally low levels of nitric oxide, provide important insights and research opportunities regarding how red blood cell "stickiness," vessel inflammation, and vasoconstriction all combine to disrupt blood flow.

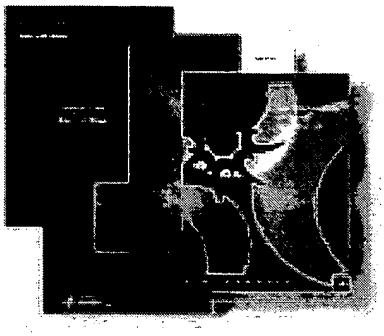
Summary

The story of the National Sickle Cell Disease Program is a story of progress and promise. The progress is reflected in a doubling of the life expectancy for patients with sickle cell disease since 1970, with patients now living on average into their mid-forties. It is reflected in the successful conclusion of a number of major clinical trials and in the three revisions of the NHLBI publication on managing sickle cell disease that have been necessitated by the trial

Increases in Life Expectancy of Patients with Sickle Cell Anemia



The life expectancy of patients with sickle cell anemia has doubled since the passage of the National Sickle Cell Anemia Control Act.



The NHLBI recently released a new guide to the management of sickle cell disease (available online at www.nhlbi.nih.gov/health/prof/blood/sickle/index.htm).

findings since the document was first published in 1982. It is reflected in the routine screening of infants for sickle cell disease and in the routine administration shortly after birth of antibiotic treatment to those infants identified with the disease. And it is reflected in the FDA approval of hydroxyurea as a proven therapy to reduce the painful, debilitating, and damaging sickle cell crises in adults.

The promise is that the NHLBI will continue to support and encourage research to improve the lives of persons with sickle cell disease. We will support and encourage new basic research studies to advance our fundamental understanding of the disease that will provide the insight needed to develop new approaches to treatment and cure. We will support and encourage followup clinical investigations of basic science advances to determine whether they are likely to benefit sickle cell patients. We will support and encourage efforts to ensure that proven treatments are rapidly disseminated and applied to improve the health of sickle cell patients. And we will not consider the National Sickle Cell Disease Program to be a complete success until babies born with sickle cell disease enjoy the same life expectancy and the same quality of life as everyone else.

1: Blood. 1987 Apr;69(4):1109-13.

Links

Fetal hemoglobin-containing cells have the same mean corpuscular hemoglobin as cells without fetal hemoglobin: a reciprocal relationship between gamma- and beta-globin gene expression in normal subjects and in those with high fetal hemoglobin production.

Dover GJ, **Boyer SH**.

We have developed methodology that allows comparison of the mean corpuscular hemoglobin (MCH) of fetal hemoglobin (HbF)-containing red cells (F cells) with the MCH of non-F cells from the same individual. To do this, suspensions of peripheral blood erythrocytes and their internal contents are fixed with an imidodiester, dimethyl-3,3'-dithiobispropionimidate dihydrochloride (DTBP). Thereafter fixed cells are made permeable to antisera by treatment with Triton X-100 and isopropanol, reacted with a mouse monoclonal antibody (MoAb) against HbF, and then with fluorescein-conjugated antimouse IgG. No appreciable hemoglobin is lost during such manipulation. Red cells from a diversity of subjects were thus treated and examined microscopically, first by transmitted light and then by epifluorescence. A direct correlation between Coulter-derived MCH and mean absorbance of 415 nm transmitted light was found for 100 unfixed (r = 0.96) and for 100 antibody-treated fixed-permeabilized red cells (r = 0.99) among individuals selected so as to provide a range of Coulter MCH values between 20 and 35. Comparisons of microscopically derived MCH of F cells and non-F cells were statistically nondistinguishable (P greater than 0.05) in all subjects. Such comparisons included normal individuals (less than 1% F cells), SS patients (7% to 48% F cells), subjects with congenital anemia (22% to 65% F cells), individuals with heterocellular hereditary persistence of HbF (HPFH) (12% to 21% F cells), and heterozygotes for beta + thalassemia (11% to 31% F cells). We conclude that gamma- and beta-globin production within F cells is regulated in a reciprocal fashion both among normal individuals and among individuals with elevated HbF production.

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